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# Effects of ATP analogs on the proton pumping by the vacuolar H<sup>+</sup>-ATPase from maize roots

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Understanding the regulatory properties of the activities of the V-type adenosine triphosphatase (ATPase) on tonoplast membranes is important in determining the mechanisms by which this enzyme controls cytoplasmic and vacuolar pH. The possible existence of a regulatory site for adenine nucleotides was examined by comparing the effects of ADP, adenylylimidodiphosphate (AMP-PNP) and 3'-*o*-(4-benzoyl) benzoyladenine 5'-triphosphate (BzATP) to those of the 2',3'-dialdehyde derivative of AMP (oAMP) and ATP by using highly purified tonoplast vesicles from maize (*Zea mays* L. cv. FRB 73) roots. The addition of either AMP-PNP or BzATP reversibly inhibited the initial rate of proton transport catalyzed by the H<sup>+</sup>-ATPase in a concentration-dependent manner. Less than 20  $\mu$ M AMP-PNP or 50  $\mu$ M BzATP was sufficient to inhibit half the initial rate of proton transport in the presence of 2 mM ATP and an excess of Mg. Both analogs increased the  $K_m$  for ATP and reduced the maximum enzyme velocity. The presence of ADP also inhibited proton transport. The characteristics of ADP-induced inhibition were similar to those of BzATP and AMP-PNP. The addition of the periodated derivative of AMP (oAMP) irreversibly inhibited the ATPase in a concentration and time-dependent manner similar to that reported previously (Chow et al. 1992, *Plant Physiology* 98: 44–52). Irreversible inhibition by oAMP reduced the maximum velocity of the tonoplast ATPase and was prevented by the addition of ATP. The presence of ADP, AMP-PNP or BzATP had no effect on irreversible inhibition by oAMP. The effects of ADP, AMP-PNP and BzATP on the kinetics of ATP utilization and the lack of protection against inhibition by oAMP argue in favor of at least two types of nucleotide binding sites on the V-type ATPase from maize root tonoplast membranes.

**Key words** – Competitive inhibition, corn, noncompetitive inhibition, nucleotide, regulation, *Zea mays*.

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## Introduction

Enzymes that link the transport of ions to either the hydrolysis or synthesis of ATP have been classified into three broad categories of transport adenosine triphosphatases (ATPases), P, V- and F-type (Nelson and Taiz 1989). These three types of ATPases can be distinguished by their biochemical, physical and physiological properties. Under most physiological conditions, the F-type ATPase couples the flux of ions, usually protons, down its electrochemical gradient to the synthesis of ATP. The other two

classes of ATPases couple the free energy from the hydrolysis of ATP to the transport of ions against their electrochemical gradient.

The mechanism by which the energy from ATP is converted into the movement of ions by the V- and P-type ATPases is poorly understood. A generalized reaction scheme for the P-type ATPases consistent with a direct coupling mechanism between ATP hydrolysis and ion transport has been proposed for many years (Skou 1965). According to this type of model, transport reactions are tightly associated with reactions leading to phosphoryla-

tion and dephosphorylation of the enzyme (Jorgensen and Andersen 1986). Soon after the discovery of V-type ATPase, it was hypothesized that ATP hydrolysis and ion transport were not tightly linked, since ATP hydrolysis and ion transport activities of V-type ATPases were differentially inhibited or activated by various treatments (Tu et al. 1987, 1990, 1992). Thus, an indirect coupling hypothesis was proposed for V-type ATPases. According to an indirect coupling mechanism, the ATPase is composed of three functional domains, one leading to ATP hydrolysis and another to ion transport. These two sets of reactions do not share a common intermediate but rather are linked by a third domain, the coupling reactions. Recent evidence indicates that the coupling between ATP hydrolysis and ion transport by P-type ATPases may not be as rigid as initially envisioned (Brauer et al. 1991, Cornelius 1990, Ikemoto et al. 1981, Inesi and deMeis 1989, Tu et al. 1992), and a reaction mechanism based on indirect coupling may account for certain data better than a direct coupling mechanism.

Biochemical studies of the V-type ATPase thus far have focused on the two predominant peripheral subunits, A and B, having molecular weights of 67 to 72 kDa and 52 to 62 kDa, respectively, and the proteolipid that binds N, N'-dicyclohexylcarbodiimide (DCCD) (Sze 1984). Analyses of the genes that code for subunits A and B indicate that these two genes probably arose from a gene duplication event (Nelson and Taiz 1989). Both genes contain an ATP binding motif, indicating that both proteins could bind the substrate ATP.

Evidence that the subunit A contains the catalytic site for ATP hydrolysis comes from several studies utilizing inhibitors. The reagent 4-chloro-7-nitrobenz-2-oxa-1,3-diazole (NBD-Cl) was identified as a potent inhibitor of the tonoplast ATPase (Mandala and Taiz 1986, Randall and Sze 1986). The presence of ATP reduced the degree of inhibition and labeling induced by NBD-Cl, suggesting that this reagent reacted at an ATP binding site (Mandala and Taiz 1986, Randall and Sze 1986). The subunit labeled by NBD-Cl was the subunit A (72 kDa). These results indicate that the polypeptide A contained the ATP catalytic site.

Further evidence for the existence of the catalytic site for ATP on the polypeptide A comes from the research of Chow et al. (1992) with the 2',3'-dialdehyde derivative of ATP (oATP). The derivative oATP inhibited the tonoplast ATPase from mung bean in time- and concentration-dependent fashion, consistent with the incorporation of 1 mol of oATP per inactivated ATPase complex. The oATP inhibited the ATPase in a manner competitive with that of ATP. When the ATPase was incubated with radioactive oATP, the label was incorporated into the polypeptide A (70 kDa). At present, the preponderance of the data suggests that the subunit A contains the catalytic site for ATP hydrolysis.

What role, if any, does the ATP binding motif on the B subunit have? Manolson et al. (1985) speculated that the B subunit had an ATP regulatory site, based on the

characteristics of inhibition by the ATP analog 3'-o-(4-benzoyl)benzoyladenine 5'-triphosphate (Bz-ATP). Upon photoactivation, BzATP covalently modified the V-type ATPase from higher plants. Incorporation of BzATP into the enzyme was competitively inhibited by ATP and demonstrated saturation kinetics, suggesting that the modifier was acting at the ATP catalytic site. However, incorporation of  $^{32}\text{P}$  from BzATP exclusively labeled subunit B and not subunit A, the presumed site for ATP catalysis. The addition of BzATP altered the kinetics with respect to ATP in a very complex manner, suggesting that BzATP acted at a regulatory site. Further data seem to be needed to establish the existence of multiple nucleotide sites on the plant V-type ATPase. The objective of these experiments was to determine the characteristics of inhibition of the V-type ATPase from maize root tonoplast vesicles by a variety of compounds with structures similar to that of ATP. The ATP analog adenylylimidodiphosphate (AMP-PNP) was chosen because it is similar in structure to that of ATP but is not hydrolyzed, while BzATP was utilized to confirm the work of Manolson et al. (1985).

**Abbreviations** – AMP-PNP, adenylylimidodiphosphate; AO, acridine orange;  $A_s$ , extent of proton transport as measured by the steady-state change in absorbance of acridine orange; BTP, bis-tris-propane; BzATP, 3'-o-(4-benzoyl)benzoyladenine 5'-triphosphate;  $\text{H}^+$ -ATPase, proton translocating adenosinetriphosphatase;  $\text{H}^+$ -PPase,  $\text{H}^+$ -translocating pyrophosphatase; NBD-Cl, 4-chloro-7-nitrobenz-2-oxa-1,3-diazole; oAMP, periodated treated AMP or 2',3'-dialdehyde derivative of AMP.

## Materials and methods

### Preparations of membrane fractions

Corn (*Zea mays* L. cv. FRB 73) seeds were germinated on filter paper moistened with 0.1 mM  $\text{CaCl}_2$  for 3 days at 28 to 30°C and harvested as described previously (Nagahashi and Baker 1984). Highly purified tonoplast vesicles were purified by differential and sucrose density centrifugation essentially as described previously (Tu et al. 1987). Tonoplast prepared from roots of cultivar FRB 73 by this protocol are essentially free of other endomembranes based on marker enzymes (Tu et al. 1987). Protein concentration was determined after precipitation by trichloroacetic acid in the presence of deoxycholate by the Lowry method (Bensadon and Weinstein 1976).

### Assays for proton pumping and phosphate appearance

Proton transport by the vacuolar  $\text{H}^+$ -ATPase was assayed by changes in acridine orange (AO) absorbance at 492.5 nm as described previously (de Michelis et al. 1983). One hundred- $\mu\text{l}$  aliquots of vesicles were diluted to 2.0 ml with assay medium containing 17.5 mM 2-(N-morpholino)ethanesulfonic acid (MES) titrated to pH 6.45 with bis-tris-propane (BTP), 0.2 mM ethylene glycol-bis (B-aminoethylether) N, N, N', N'-tetraacetic acid, 2.5 mM  $\text{MgSO}_4$ , 7.5  $\mu\text{M}$  AO and 50 mM KCl. After incubation at

18 to 24°C for 5 min, proton transport was initiated by the addition of 20  $\mu\text{l}$  of 0.2 M ATP titrated to pH 6.45 with BTP. The initial rate of proton transport was determined by analyzing the time course of quenching of AO absorbance by a model that considers net proton transport as the difference between the rate of pumping by the transporter and the summation of the inhibitory processes including proton leakage and pump slippage as described previously (Brauer et al. 1989, Tu et al. 1987). The standard error of the mean is presented as a bar through data points only in those cases in which the error is greater than the data symbol.

#### Preparation of inhibitors and experimental conditions

Solutions of AMP-PNP and oAMP were prepared in distilled water immediately before use. Nucleotides, AMP-PNP, oAMP and BzATP were obtained from Sigma. Modification of the tonoplast  $\text{H}^+$ -ATPase by oAMP was conducted by the protocol of Chow et al. (1992).

In certain experiments, the effects of various nucleotides or nucleotide analogs on the time-dependent, irreversible inactivation of the  $\text{H}^+$ -ATPase by oAMP were assessed using the following protocol. An aliquot of tonoplast vesicles was adjusted to 1 mM oAMP in the absence of other compounds or in the presence of 2 mM ATP, 0.3 mM ADP, 0.2 mM AMP-PNP or 0.2 mM BzATP. Immediately after they were mixed, 100- $\mu\text{l}$  aliquots were removed and diluted to 2.0 ml with AO assay media to measure proton transport to estimate the activity present before covalent modification by oAMP. Additional 100- $\mu\text{l}$  aliquots were removed over the next 40 min to assay the proton transport activity remaining after possible modification by oAMP. The dilution of the tonoplast vesicles 20-fold in AO assay media greatly reduced the level of inhibition of proton transport by ADP, AMP-PNP or BzATP present during the incubation with oAMP.

## Results

#### Initial characterization of inhibition by AMP-PNP and BzATP

When tonoplast vesicles were supplied with 0.02 to 2 mM AMP-PNP or BzATP in the absence or presence of 2.5 mM Mg, there was no enzymatic liberation of phosphate over a 20 min period (data not shown), indicating that neither of these ATP analogs were hydrolyzed to any great extent. The addition of either AMP-PNP or BzATP was found to decrease the proton transport activity of the tonoplast  $\text{H}^+$ -ATPase (Fig. 1). Inhibition by both compounds was concentration dependent. Approximately either 20  $\mu\text{M}$  AMP-PNP or 50  $\mu\text{M}$  BzATP was necessary to inhibit half the initial rate of proton transport. The commercial preparations of AMP-PNP contain significant amounts of lithium and phosphate, whereas BzATP was supplied as the tetraethylammonium salt. The addi-

tion of lithium, tetraethylammonium or phosphate up to 10 mM had no effect on proton transport activity (data not shown). Thus, the effects of BzATP and AMP-PNP were attributed to those of the nucleotide derivative.

Further characterization of the inhibition of the tonoplast  $\text{H}^+$ -ATPase was accomplished by comparing the kinetics of proton transport with respect to ATP (Fig. 2). In the presence of either AMP-PNP or BzATP, both the  $K_m$  for ATP and the maximum velocity of proton transport were affected. The  $V_{\max}$  was 67 and 41% of the value obtained in the absence of AMP-PNP in the presence of 5 and 20  $\mu\text{M}$  AMP-PNP, respectively. The  $K_m$  for ATP increased from 0.27 mM to 0.35 and 0.50 mM in the presence of 5 and 20  $\mu\text{M}$  AMP-PNP, respectively. The presence of BzATP had similar effects on the kinetics of ATP-dependent proton transport. The addition of 40  $\mu\text{M}$  BzATP increased the  $K_m$  to 0.41 mM and decreased the  $V_{\max}$  to 50% of the value obtained in the absence of BzATP. Since both  $V_{\max}$  and  $K_m$  were affected, AMP-PNP and BzATP were not simple competitive inhibitors of the tonoplast ATPase.

#### Effects of ADP

While ADP is not a substrate of the vacuolar proton pump (Tu et al. 1987), it has been shown to be a competitive inhibitor of ATP hydrolysis by the tonoplast  $\text{H}^+$ -ATPase (Griffith et al. 1986). It was of interest to determine the characteristics of ADP inhibition on proton transport catalyzed by the vacuolar proton pump because the sensitivity and characteristics of inhibition of the proton transport activity of the vacuolar proton pump from maize roots can be different from those observed for the ATP hydro-

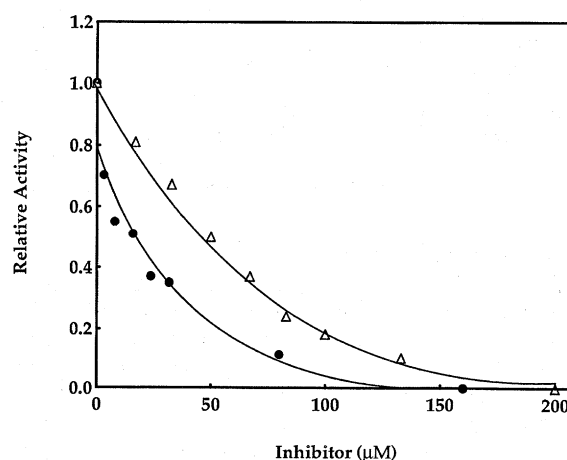


Fig. 1. Effects of AMP-PNP and BzATP on the initial rate of proton transport by the tonoplast  $\text{H}^+$ -ATPase from maize roots. Either AMP-PNP ( $\bullet$ ) or BzATP ( $\Delta$ ) was added to the reaction mixture immediately before the tonoplast vesicles were added, and proton transport was assayed by changes in AO absorbance as described in Materials and methods. Data are the average of two experiments, each with three replicates. Results are plotted relative to the rate obtained in the absence of either AMP-PNP or BzATP, which averaged  $1.05 \pm 0.03 \text{ A (mg protein)}^{-1} \text{ min}^{-1}$ .

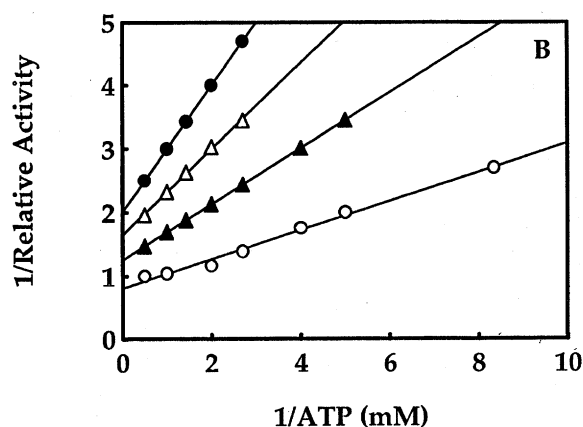
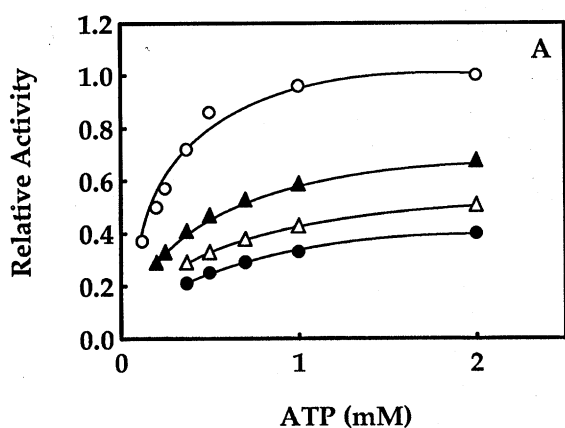


Fig. 2. Effects of AMP-PNP and BzATP on the ATP dependence of the initial rate of proton transport catalyzed by the tonoplast  $H^+$ -ATPase from maize roots. The initial rate of proton transport was determined in the absence ( $\circ$ ) and presence of  $5 \mu M$  AMP-PNP ( $\blacktriangle$ ),  $20 \mu M$  AMP-PNP ( $\bullet$ ) or  $40 \mu M$  BzATP ( $\triangle$ ). Data are plotted relative to the activity in the absence of AMP-PNP and the presence of  $2 mM$  ATP, which averaged  $1.1 \pm 0.08 A (mg \text{ protein})^{-1} \text{ min}^{-1}$ . Data are the average of two experiments each with two replicates. Data were transformed and plotted according to the Lineweaver-Burk equation in panel B.

lysis activity (Tu et al. 1987, 1990, 1992). The presence of ADP in the normal assay media containing  $2 mM$  ATP inhibited proton transport activity. At  $60 \mu M$  ADP, the maximum initial rate of proton transport was about one-half that found in its absence (Fig. 3). The effects of ADP on the kinetics with respect to ATP indicated that the ADP was not a simple competitive inhibitor of proton transport since both the  $K_m$  for ATP and the  $V_{max}$  were altered (Fig. 3B). Addition of  $60$  and  $100 \mu M$  ADP increased the  $K_m$  for ATP from  $0.26 mM$  to  $0.50$  and  $0.80 mM$  ATP, respectively. The  $V_{max}$  decreased by approximately  $50$  and  $70\%$  of that obtained in the absence of ADP in the presence of  $60$  and  $100 \mu M$  ADP, respectively. Therefore, inhibition of proton transport by the vacuolar  $H^+$ -ATPase by ADP was similar to that observed with AMP-PNP and BzATP, but different from the effects

of ADP on ATP hydrolysis by the vacuolar  $H^+$ -ATPase from *Beta vulgaris* (Griffith et al. 1986).

#### Effects of oAMP

Chow et al. (1992) provided convincing evidence that the periodated derivatives of adenosine nucleotides inhibit the tonoplast ATPase by covalently modifying the enzyme at the ATP catalytic site. In the present study the adenosine monophosphate derivative (oAMP) was chosen because results from another study in our laboratory (data not shown) indicated that this compound had better stability and fewer contaminants than the other two periodated derivatives of adenosine nucleotides. The presence of oAMP under conditions of minimal covalent modification of the tonoplast pump inhibited the enzyme

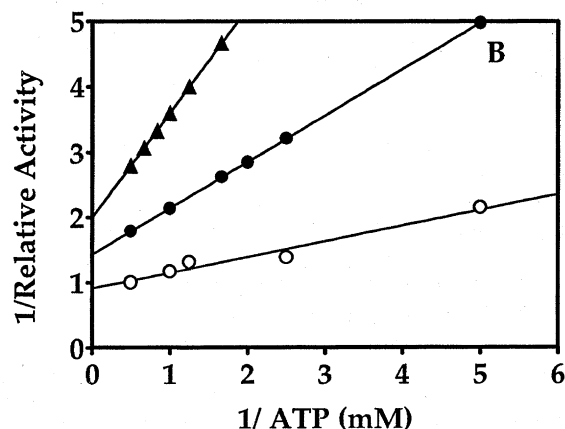
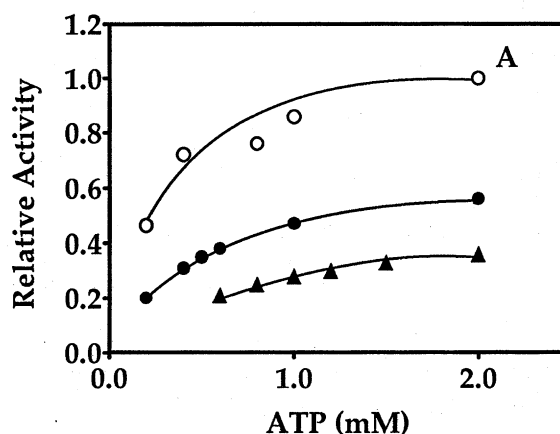


Fig. 3. Effects of ADP on the ATP dependence of the initial rate of proton transport catalyzed by tonoplast  $H^+$ -ATPase from maize roots. Initial rate of proton transport was determined in the absence ( $\circ$ ) and presence of either  $60$  ( $\bullet$ ) or  $100 \mu M$  ADP ( $\blacktriangle$ ). In panel A, data are plotted relative to the activity in the absence of ADP and presence of  $2 mM$  ATP, which averaged  $1.05 \pm 0.07 A (mg \text{ protein})^{-1} \text{ min}^{-1}$ . Data were transformed and plotted according to the Lineweaver-Burk equation in panel B. Data are the average of two experiments, each with two replicates.

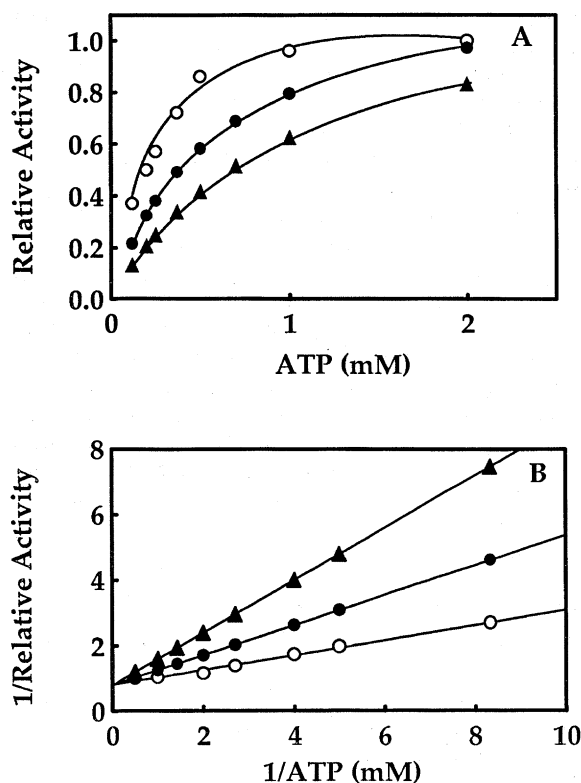


Fig. 4. Effects of oAMP on the ATP dependence of the initial rate of proton transport catalyzed by the tonoplast  $H^+$ -ATPase from maize roots. The initial rate of proton transport was determined in the absence ( $\circ$ ) and presence of either 1 ( $\bullet$ ) or 2 mM oAMP ( $\blacktriangle$ ). The reaction mixture was adjusted to the appropriate oAMP concentration immediately before the initiation of transport by the addition of ATP to minimize covalent modification of the proton pump by oAMP. Data are plotted in panel A relative to the rate obtained in the absence of oAMP and presence of 2 mM ATP, which averaged  $1.08 \pm 0.05$  A (mg protein) $^{-1}$  min $^{-1}$ . Data were transformed according to the Lineweaver-Burk equation and plotted in panel B.

activity in a manner that was competitive with respect to ATP (Fig. 4). The oAMP inhibited the maize root vacuolar ATPase in a time- and concentration-dependent fashion, consistent with covalent modification of the enzyme (Fig. 5). When the ATPase was covalently modified by oAMP, the  $K_m$  of the remaining ATPase was essentially the same as that of untreated ATPase (data not shown). Inhibition by oAMP was substantially reduced by the presence of ATP (Fig. 5). Therefore, the results with oAMP from this lab were similar to those reported previously by Chow et al. (1992) and consistent with the hypothesis that oAMP inhibited the ATPase by acting at or near the ATP catalytic site. Inhibition of proton transport by oAMP was associated with decreases in the extent of proton transport (data not shown).

#### Test for an interaction between oAMP and other nucleotide analogs

If the presence of ADP, AMP-PNP or BzATP inhibited the tonoplast ATPase by binding at or near the catalytic site for ATP, the addition of these compounds should protect activity from time-dependent inactivation by oAMP in a manner similar to that of the substrate ATP. The addition of 1 mM oAMP resulted in a time-dependent inhibition in the initial rate of proton transport (Fig. 5). In the presence of a saturating level of ATP, little inhibition was observed. Presumably, the ATP competed with oAMP for binding and thus prevented inhibition. Inhibition of the proton transport activity by 1 mM oAMP was also conducted in the presence of 0.2 mM AMP-PNP, 0.3 mM ADP or 0.2 mM BzATP. These concentrations were chosen because they were the minimal levels necessary to induce total inhibition of the proton transport activity when they were added to the reaction media, but substantial activity could be recovered when the concentrations were reduced by dilution. The presence of either AMP-PNP, ADP or BzATP during the oAMP treatment had little or no effect on the time dependence of oAMP inhibition.

#### Discussion

The results presented here are consistent with the presence of at least two types of nucleotide binding sites on the V-type ATPase from maize root tonoplast vesicles. At one of these sites, the substrate ATP and the inhibitor oAMP competed for binding (Fig. 5). This site is presumed to be the one that catalyses ATP hydrolysis. There appeared to be at least one other nucleotide site which bound ADP, AMP-PNP and BzATP. Evidence that this

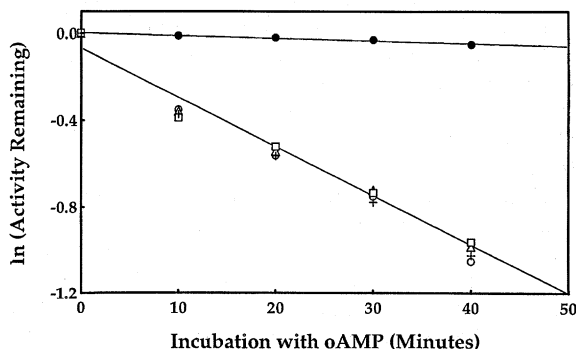


Fig. 5. Effect of AMP-PNP, ATP, ADP and BzATP on the time course of inhibition induced by oAMP. Tonoplast vesicles were incubated with 1 mM oAMP for 0, 10, 20, 30 or 40 min at 18 to 22°C in the absence ( $\circ$ ) and presence of 0.2 mM AMP-PNP ( $\triangle$ ), 2 mM ATP ( $\bullet$ ), 0.3 mM ADP ( $\square$ ) or 0.2 mM BzATP ( $+$ ). Proton transport was assayed after the above incubation times as described in Materials and methods. Data are plotted relative to the activity at zero time which averaged  $1.02 \pm 0.05$ ,  $0.58 \pm 0.03$ ,  $1.03 \pm 0.04$ ,  $0.79 \pm 0.06$  and  $0.72 \pm 0.04$  A (mg protein) $^{-1}$  min $^{-1}$  in the absence and presence of AMP-PNP, ATP, ADP or BzATP, respectively. Data are the average of two experiments each with three replicates.

site existed and was distinct from that of the catalytic site was twofold. First, the presence of ADP, AMP-PNP and BzATP inhibited that proton transport activity in a manner dissimilar to that of the competitive inhibitor, oAMP. Second, the presence of these three nucleotide analogs did not prevent the time-dependent inactivation of the ATPase by oAMP, as did the substrate (Fig. 5). It is not certain whether the BzATP, AMP-PNP and ADP bind to one site or several distinct sites. The interaction between the three inhibitors needs to be examined more thoroughly before a definite conclusion can be reached.

The evidence described in the present work that ADP is a noncompetitive inhibitor of the tonoplast H<sup>+</sup>-ATPase does not necessarily contradict earlier reports that ADP is a competitive inhibitor of the tonoplast H<sup>+</sup>-ATPase. One line of evidence that ADP interacted at the catalytic site for ATP, and thus was a competitive inhibitor of the H<sup>+</sup>-ATPase, comes from experiments examining the interaction between NBD-Cl and ADP (Randall and Sze 1987, Yamanishi and Kasamo 1992). Both of these studies demonstrated that NBD-Cl inactivated the tonoplast H<sup>+</sup>-ATPase in a manner consistent with the covalent modification of cysteine involved in the binding of the substrate, ATP. The addition of ADP prevented NBD-Cl inactivation. However, millimolar concentrations of ADP concentration were necessary to overcome the effects of NBD-Cl. In the present study, much lower concentrations of ADP were effective inhibitors of proton transport. For example, 0.1 mM ADP was able to reduce the maximum rate of proton transport by 70% (Fig. 3). Therefore, inhibition of the H<sup>+</sup>-ATPase by ADP could occur at low concentrations (< 0.2 mM) by interaction at a site other than the one involved in ATP hydrolysis. At higher ADP concentrations, the interaction between the proton pump and ADP could occur at the catalytic site for ATP.

A second line of evidence that ADP is a competitive inhibitor of the H<sup>+</sup>-ATPase is presented by Rausch et al. (1985), who examined the effects of ADP on ATP kinetics of vacuolar-type proton transport in microsomal vesicles isolated from maize coleoptiles and roots. Rausch et al. (1985) contend that ADP is a competitive inhibitor of the tonoplast H<sup>+</sup>-ATPase from both tissues with half maximal inhibition occurring between 0.1 and 0.2 mM ADP. The data from roots are difficult to interpret as presented in the 1985 manuscript. First, the figure legend states that the data are plotted as an Eadie-Hofstee plot. However, the X- and Y-axis legends are not compatible with those of an Eadie-Hofstee plot. The data in the presence of 0, 0.2 or 0.4 mM ADP are fitted to three lines that share a common Y-intercept. However, the Y-intercepts from the three lines are different when regression equations are generated from the data points. Our analyses of the published data predict that the Y-intercept decreases from 6.2 to 5.2 when data from controls are compared to those obtained in the presence of 0.2 mM ADP. Such a decrease in the Y-intercept in an Eadie-Hofstee plot is more consistent with noncompetitive inhibition than competitive inhibition. The regression equation for the data in the

presence of 0.4 mM ADP had a low correlation coefficient ( $r = 0.4$ ), making a valid estimate of the Y-intercept almost impossible. In contrast, the assertion that ADP is a noncompetitive inhibitor of the maize root tonoplast H<sup>+</sup>-ATPase in the present study is based on appearance of the raw data and statistically significant regression equations of transformed data.

The existence of a site on the tonoplast ATPase that binds ADP and inhibits proton transport activity may have interesting physiological significance. In a recent publication from our laboratory, Brauer et al. (1992) proposed that the proton-translocating pyrophosphatase (H<sup>+</sup>-PP<sub>ase</sub>) was the mechanism by which the trans-vacuolar pH gradient was maintained under conditions of low cytoplasmic ATP in corn roots. One role for the ADP inhibitory site on the tonoplast H<sup>+</sup>-ATPase may be to reduce the ATP-dependent proton transport rate further than that which would have occurred by the reduction in substrate. The advantage to the cell would be that the magnitude of the loss of protons via the inhibitory pathways would be less. The characteristics of proton transport and the inhibitory pathway are such that rate constants describing the loss of protons from the vesicular lumen are less when the H<sup>+</sup>-PP<sub>ase</sub> is generating the gradient than when the H<sup>+</sup>-ATPase is operating (Brauer et al. 1992). Therefore, a complete shutdown of the H<sup>+</sup>-ATPase by ADP could lead to a reduction in the loss of transport protons from the vacuolar lumen and thus help to maintain the transvacuolar pH gradient under conditions of low ATP and high ADP.

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